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The influence of residue 190 in the S1 site of trypsin-like serine proteases on substrate selectivity is universally conserved

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Abstract We examined the influence of Ser/Ala190 in the S1 site on P1 substrate selectivity in several serine proteases. The impact of residue 190 on the selectivity was constant, regardless of differences in original selectivity or reactivity. Substrate binding in S1 was optimised in all wild-type enzymes, while the effects on k_{cat} depended on the combination of residue 190 and substrate. Mutagenesis of residue 190 did not affect the S2–S4 sites. Pronounced selectivity for arginine residues was coupled with low enzymatic activity, in particular in recombinant factor IXa. This is due to the dominance of the S1–P1 interaction over substrate binding in the S2–S4 sites.

Key words: Activity modulation; Activity profile; Coagulation enzyme; Induced fit; Hybrid protease; Site-directed mutagenesis

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1. Introduction

All trypsin-like serine proteases share a substrate preference for a basic P1 residue, lysine or arginine. This is mainly caused by the presence of a negatively charged Asp189 at the bottom of the S1 pocket (chymotrypsinogen numbering used throughout) [1]. The architecture of the S1 site within these proteases is highly conserved [2]. One marked difference is localised to position 190, which is either serine or alanine, and serves as a fingerprint of the enzyme subfamilies. Significantly, the enzymes of the blood coagulation cascade differ in this position. The executioners of the cascade, thrombin and factor Xa, have Ala190, whereas the physiologic activators of factor X, factor VIIa and factor IXa, have serine conserved at position 190 through different species [3,4].

This observation poses the question whether Ser/Ala190 translates into the different substrate specificity of these enzymes and whether this difference affects substrate residues in addition to the P1 residue. The P1 residue of almost all physiologic substrates of the coagulation factors VIIa, IXa, Xa and thrombin is arginine [5]. In the absence of the physiological protein cofactor and substrate, factor VIIa and in particular factor IXa are very poor enzymes [6,7]. This property might also be linked to the primary specificity of these enzymes.

From a structural point of view, the binding of arginine into the S1 site differs from lysine as only the former is able to form a direct ionic interaction with Asp189, while the interaction of the shorter lysine side chain to Asp189 is water-bridged [8]. Ser190 is positioned in the S1 pocket to allow an additional hydrogen bond to a bound lysine or arginine, thus stabilising either of the substrate residues [9,10].

We considered the various S1–P1 interactions as a binary 2×2 matrix with the enzyme having either serine or alanine at position 190 and the substrate having either arginine or lysine at P1. To systematically investigate this simplified system, we introduced point mutations at position 190 (Ala ↔ Ser) in the coagulation factors rf9a (Ser190) and rf10a (Ala190), trypsin (Ser190), and rXY (Ser190), a hybrid containing the N-terminal subdomain of factor Xa and the C-terminal subdomain of trypsin (wild-type residue is given in parentheses) [11]. For each of these four proteases we kinetically analysed both the wild-type and the appropriate mutant enzyme using commercially available *para*-nitroanilide (pNA) substrates.

2. Materials and methods

2.1. Chemicals

All synthetic substrates were purchased from Roche Diagnostics (Mannheim, Germany) and Chromogenix (Mölndal, Sweden). All other materials were of the highest grade commercially available.

2.2. Construction and production of recombinant proteins

All wild-type and mutant proteins were from human species and produced according to previously published procedures [11–13]. Trypsin denotes the predominant pancreatic trypsin I isoform.

2.3. Amidolytic assays and determination of kinetic parameters

Experiments were performed in 50 mM Tris, 150 mM NaCl, 5 mM $CaCl_2$, 0.1% PEG 8000, pH 8.0 at 25°C in a temperature-controlled Kontron-Uvikon 933 spectrophotometer (Kontron Instruments, Milan, Italy). Enzyme concentrations were between 1 and 1000 nM. Seven different substrate concentrations of a two-fold dilution series were used. Depending on the substrate and enzyme, the starting concentrations of the substrate ranged between 0.5 and 10 mM. Reactions were started by adding the enzyme to the pre-warmed reaction buffer. The change in absorption at 405 nm was monitored for typi-

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Abbreviations: rf9a, recombinant factor IXa; rf10a, recombinant factor Xa; rTry, recombinant trypsin; rXYa, recombinant factor Xa-trypsin hybrid; pNA, *para*-nitroanilide; tGPR, Chr TH; tGPK, Chr PL; tGPX, substrate pair tGPR and tGPK; VLR, S-2266; VLK, S-2251; VLX, substrate pair VLR and VLK

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Table 1
Kinetic parameters for the hydrolysis of a series of peptide substrates by rTry Ser/Ala190, rXYa Ser/Ala190 and rf10a Ala/Ser190

Substrate						rTry Ser190 (WT)			rTry Ala190			rXya Ser190 (WT)			rXYa Ala190			rf10a Ala190 (WT)			rf10a Ser190		
	(P4)	P3	P2	P1		k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})
S-2444	Pyro-	Glu-	Gly-	Arg-	pNA	184.1	159.7	1.15	281.3	388.1	0.72	47.3	37.8	1.25	95.0	137	0.69	–[a]	–	0.0310	–	–	0.0151
S-2222	Bz-Ile-	Glu-	Gly-	Arg-	pNA	138.0	57.5	2.40	233.2	109.1	2.14	43.5	16.3	2.67	113.1	91	1.24	95.8	149	0.6445	59.7	230	0.2592
Chr t-PA	MS-	D-Phe-	Gly-	Arg-	pNA	167.6	30.4	5.51	211.4	51.0	4.15	46.3	9.3	4.96	130.5	40	3.25	121.3	385	0.3152	72.0	491	0.1465
Chr X	MOC-	D-Nle-	Gly-	Arg-	pNA	157.5	32.7	4.82	192.6	56.4	3.42	54.3	13.2	4.12	133.5	50	2.66	163.0	209	0.7788	99.9	251	0.3982
Chr U	Bz-	β-Ala-	Gly-	Arg-	pNA	91.6	132.3	0.69	146.1	412.9	0.35	38.0	49.2	0.77	88.0	175	0.50	29.3	475	0.0616	34.0	1315	0.0258
Chr Try	CB-	Val-	Gly-	Arg-	pNA	103.0	53.9	1.91	123.7	121.0	1.02	32.9	12.4	2.65	69.6	41	1.71	59.8	639	0.0936	58.0	1525	0.0380
Chr PK	Bz-	Pro-	Phe-	Arg-	pNA	11.2	150.5	0.07	14.4	352.9	0.04	32.0	69.8	0.46	62.0	263	0.24	11.1	965	0.0115	6.0	1327	0.0045
Chr GK		D-Val-	CHA-	Arg-	pNA	30.4	110.0	0.28	61.6	330.1	0.19	58.8	38.5	1.53	107.6	158	0.68	35.1	1067	0.0329	37.2	2343	0.0159
Chr TH	Tos-	Gly-	Pro-	Arg-	pNA	106.7	16.5	6.48	145.8	30.8	4.74	35.6	23.5	1.51	82.3	92	0.89	71.8	203	0.3534	56.3	336	0.1673
Chr PL	Tos-	Gly-	Pro-	Lys-	pNA	19.1	13.5	1.42	27.0	137.8	0.20	70.0	116.0	0.60	32.2	633	0.05	37.7	966	0.0391	111.5	1820	0.0613
S-2266		D-Val-	Leu-	Arg-	pNA	26.6	139.0	0.19	67.0	603.7	0.11	27.1	117.8	0.23	80.5	642	0.13	–	–	0.0187	36.8	3859	0.0095
S-2251		D-Val-	Leu-	Lys-	pNA	11.6	499.1	0.0233	5.18	2176.0	0.0024	75.2	1133	0.0663	57.9	14 030	0.0041	–	–	0.00021	–	–	0.00054

Bz, benzyl; CB, carbobenzoxy; CHA, cyclohexylalanyl; MOC, methoxycarbonyl; MS, methylsulfonyl; Tos, t, tosyl.

[a] K_M was too high for these enzyme–substrate combinations. Therefore, it was not possible to determine k_{cat} and K_M individually.

cally 2 min and ΔE/min was calculated from the linear part of the curve with a correlation coefficient of the linear regression >0.99. Kinetic parameters were calculated by non-linear curve fitting of the initial rates to the Michaelis–Menten equation using the SigmaPlot software (Jandel, Erkrath, Germany) and an extinction coefficient for pNA of $\varepsilon_{405} = 9920\ M^{-1}\ cm^{-1}$.

3. Results and discussion

3.1. *Biochemical characterisation of the impact of Ser/Ala190 on the amidolytic activity of rTry, rXYa, rf10a*

The kinetic parameters k_{cat}, K_m and k_{cat}/K_m were determined for rTry, rXYa, rf10a and the respective mutants in position 190 using a series of commercially available pNA peptide substrates (Table 1). The majority of these substrates had an arginine in P1 and differed in P2–P4. Two substrate pairs differed in P1 (lysine versus arginine), while the other residues were identical.

3.2. *Mutagenesis of position 190 does not change the selectivity profile of S2–S4 in rTry, rXYa and rf10a*

It cannot be excluded that mutations in S1 have long-distance effects on S2–S4. Precedents for analogous long-range dependences of substrate specificity and reactivity are found in several proteases (e.g. [13,14]). We therefore examined the influence of the S1 site mutation in position 190 on the substrate selectivity in S2–S4 to test for such long-distance effects. This was performed by comparison of the reactivity of the mutant and wild-type enzymes with a series of substrates that were identical in P1 (arginine) but differed in S2–S4 (Table 2). In particular, we looked for every enzyme at its normalised activity profile, which is independent of the absolute reactivities and is thus better suited for comparison.

The activity with P1–arginine substrates was decreased in all mutants to about half the original value. The changes in activity were in a similar range, as reflected by similar k_{cat}/K_M ratios in Table 2, irrespective of the differences in overall reactivity with the substrates that spanned two orders of magnitude. The conservation of the activity profile between wild-type and mutant enzymes indicates that mutagenesis of residue 190 does not influence the other binding sites. Otherwise, alteration of the S2–S4 sites would lead to changed substrate preferences and cause a modified activity profile with the substrates that are identical in P1 but differ in P2–P4. This finding was also confirmed by comparison of the crystal structures of rXYa and rXYa-S190A, where no conformational rearrangement in the S2–S4 sites was observed [15].

3.3. *Position 190 has a comparable influence on the selectivity for P1-Arg/-Lys side chains in rTry, rXYa, rf10a and rf9a*

We examined the influence of serine and alanine 190 on the selectivity for arginine and lysine substrate side chains in the S1 site of rTry, rXYa, rf10a and rf9a using two substrate pairs (tGPR and tGPK; VLR and VLK) (Table 3).

The substrate pair VLX (X = R/K) was hydrolysed approximately 10-fold less efficiently than tGPX by the enzyme pairs rTry, rXYa and rf10a. The selectivity for arginine was higher with VLX in these enzymes. The reactivity of rf9a was several orders of magnitude lower than in the other enzymes but did not differ significantly between the two substrate pairs. Compared with the other enzymes, rf9a had the highest selectivity for arginine. The influence of residue 190 was comparable in all enzymes and for both substrate pairs: the selectivity for

Table 2
Change of the kinetic parameters of rTy Ser190→Ala190, rXYa Ser190→Ala190 and rf10a Ala190→Ser190 with a series of peptide substrates represented by the ratios of the kinetic parameters

Substrate						rTry mutant/WT			rXYa mutant/WT			rf10a mutant/WT		
	(P4)	P3	P2	P1		k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M	k_{cat}	K_M	k_{cat}/K_M
S-2444	Pyro-	Glu-	Gly-	Arg-	pNA	1.53	2.43	0.63	2.01	3.64	0.55	–	–	0.49
S-2222	Bz-Ile-	Glu-	Gly-	Arg-	pNA	1.69	1.90	0.89	2.60	5.62	0.46	0.62	1.55	0.40
Chr t-PA	MS-	D-Phe-	Gly-	Arg-	pNA	1.26	1.68	0.75	2.82	4.31	0.65	0.59	1.28	0.46
Chr X	MOC-	D-Nle-	Gly-	Arg-	pNA	1.22	1.72	0.71	2.46	3.81	0.65	0.61	1.20	0.51
Chr U	Bz-	β-Ala-	Gly-	Arg-	pNA	1.60	3.12	0.51	2.32	3.55	0.65	1.16	2.77	0.42
Chr Try	CB-	Val-	Gly-	Arg-	pNA	1.20	2.25	0.53	2.11	3.28	0.64	0.97	2.39	0.41
Chr PK	Bz-	Pro-	Phe-	Arg-	pNA	1.28	2.34	0.55	1.94	3.77	0.51	0.54	1.38	0.39
Chr GK		D-Val-	CHA-	Arg-	pNA	2.03	3.00	0.68	1.83	4.09	0.45	1.06	2.20	0.48
Chr TH	Tos-	Gly-	Pro-	Arg-	pNA	1.37	1.87	0.73	2.31	3.92	0.59	0.78	1.66	0.47
Chr PL	Tos-	Gly-	Pro-	Lys-	pNA	1.41	10.25	0.14	0.46	5.45	0.08	2.96	1.89	1.57
S-2266		D-Val-	Leu-	Arg-	pNA	2.52	4.34	0.58	2.97	5.45	0.55	–	–	0.51
S-2251		D-Val-	Leu-	Lys-	pNA	0.45	4.36	0.10	0.77	12.38	0.06	–	–	2.64
Average change with arginine in P1						1.57	2.47	0.66	2.34	4.14	0.57	0.79	1.80	0.45

For abbreviatons see Table 1.

arginine was approximately 5.5-fold higher in the Ala190 enzymes than in the Ser190 enzymes.

This result indicates that the influence of the γ-hydroxyl of Ser190 on substrate binding is conserved in all enzymes examined. The hydrogen bond with Ser190 is the only direct interaction of P1-lysine with the protein matrix, while P1-arginine is also stabilised via a salt bridge of its guanidinium group with the carboxylate of Asp189 at the bottom of the S1 site [9].

The differences in initial selectivity are caused by different binding affinities in the S2–S4 binding sites. With poor canonical substrate recognition in the S2–S4 sites, the substrate binding and catalysis are dictated by the S1 site, explaining the stronger dependence on the P1 residue. Therefore, in the case of rTry, rXYa and rf10a, the arginine preference is stronger with the VLX substrate pair than with the tGPX pair which employs the conformationally and entropically favourable proline in P2. In the case of rf9a, the substrate binding is almost exclusively dictated by the S1–P1 interaction which causes a 34–40-fold preference for arginine over lysine, independent from the P2–P4 residues.

For rTry, rXYa and rf10a the kinetic parameters k_{cat} and K_M were determined separately. Consistent with all substrates, the 190 mutation always caused an increase of the K_M values. This indicates that, independent of the resulting overall activity of the enzyme, each wild-type possesses a S1 site optimised for substrate binding. In particular, the weakened substrate binding to rf10a-A190S, which allows for an additional hydrogen bond within the S1 pocket, demonstrates that yet unidentified subtle effects govern the S1–P1 recognition.

Furthermore, in each enzyme the K_M values were higher with the lysine substrate of a given substrate pair than with the arginine counterpart. This is in accordance with the working hypothesis that P1-arginine is bound more tightly and therefore has a lower K_M value than P1-lysine.

The k_{cat} values depended on the combination of residue 190 and the P1 residue. With P1-lysine, they were higher in the Ser190 variants, while with P1-arginine they were higher in the Ala190 enzymes. In the Ala190 enzymes, P1-lysine is stabilised only via hydrophobic interactions and a water bridge with Asp189. This is probably not sufficient to accurately position the scissile bond in the active site. The hydrogen bond of P1-lysine with Ser190 is necessary for correct positioning and efficient turnover of the substrate. The opposite may be true for P1-arginine: it is possible that in the presence of Ser190 it is bound too tightly in a non-optimal position. Release of this restraint in Ala190 could introduce the flexibility necessary for efficient catalysis. With rTry we observed an exception to this rule: k_{cat} with tGPK was higher in rTry Ala190 than in rTry Ser190. Since K_M for tGPK in rTry Ser190 approximates the low K_M of tGPR, it is possible that it is necessary to release the tight binding for efficient catalysis.

3.4. rf9a has a very high specificity towards arginine in P1

rf9a differs from all other investigated enzymes. Its amidolytic activity is three to seven orders of magnitude lower than that of rf10a, rXYa and rTry. Anomalously high K_M values mostly explain the low reactivity of rf9a. In fact, the K_M values are higher than the solubility of the associated substrates. Therefore, we could only determine the specificity constants k_{cat}/K_M of rf9a, but not the detailed kinetic parameters k_{cat} and K_M (Table 3). Compared with the other enzymes, rf9a displays an up to 18-fold higher selectivity of arginine over lysine in P1, for both the Ser190 wild-type and the Ala190 mutant enzyme. Finally, rf9a is the only enzyme where the mutant (Ala190) has a two-fold higher activity towards arginine-containing substrates than the wild-type enzyme.

Previous investigations linked factor IXa's low amidolytic activity to its 99 loop. The loop hinders substrate binding to the S2–S4 sites, thus causing factor IXa's high K_M values [12,13,16]. The high selectivity towards arginine in P1 is presumably a direct consequence of the poor substrate binding to rf9a's S2–S4 sites. The relative contribution of the S1–P1 binding energy to the total substrate binding is much higher in the case of rf9a than in the other enzymes, and similarly the difference in binding energy of P1-arginine versus P1-lysine is more significant. In addition to the dominating contribution of the primary specificity pocket in rf9a, the relatively high selectivity for P1-arginine may also reflect the intrinsic particularities of its architecture and function. In particular, Glu219 is strictly conserved in the S1 site of factor IXa, while other trypsin-like serine proteases have Gly at this position. The conformation of this residue is favourable only to Gly. Consistent with this, the point mutation G219E in rf10a destabilised the S1 pocket [12]. When compared to lysine, the more

Table 3
Comparison of the kinetic parameters of rTry Ser/Ala190, rXYa Ser/Ala190, rf10a Ala/Ser190 and rf9a Ser/Ala190 for the hydrolysis of two P1-arginine/-lysine substrate pairs

	tGPR			tGPK			P1 selectivity	VLR			VLK			P1 selectivity
	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	Arg/Lys	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (s^{-1} μM^{-1})	Arg/Lys
rTry Ser190 (WT)	107	16	6.48	19	13	1.42	4.6	27	139	0.19	12	499	0.0233	8.2
rTry Ala190	146	31	4.74	27	138	0.20	24.2	67	604	0.11	5	2176	0.0024	46.6
rTry Ala190/rTry Ser190	1.37	1.87	0.73	1.41	10.25	0.14		2.52	4.34	0.58	0.45	4.36	0.10	
Increase in P1-arginine selectivity							5.3							5.7
rXYa Ser190 (WT)	36	24	1.51	70	116	0.60	2.5	27	118	0.23	75	1133	0.0663	3.5
rXYa Ala190	82	92	0.89	32	633	0.05	17.5	80	642	0.13	58	14030	0.0041	30.4
rXYa Ala190/rXYa Ser190	2.31	3.92	0.59	0.46	5.45	0.08		2.97	5.45	0.55	0.77	12.38	0.06	
Increase in P1-arginine selectivity							7.0							8.8
rf10a Ser190	56	336	0.17	112	1820	0.06	2.7	37	3859	0.01	–	–	0.00054	17.6
rf10a Ala190 (WT)	72	203	0.35	38	966	0.04	9.0	–	–	0.02	–	–	0.00021	91.0
rf10a Ala190/rf10a Ser190	1.28	0.60	2.11	0.34	0.53	0.64				1.96			0.38	
rf10a Ser190/rf10a Ala190	0.78	1.66	0.47	2.96	1.89	1.57				0.51			2.64	
Decrease in P1-arginine selectivity							3.3							5.2
rf9a Ser190 (WT)			1.96E-05			5.76E-07	34.1			2.45E-05			6.11E-07	40.0
rf9a Ala190			3.93E-05			2.28E-07	172.3			4.67E-05			2.42E-07	193.1
rf9a Ala190/rf9a Ser190			2.00			0.40				1.91			0.40	
Increase P1-arginine selectivity							5.1							4.8

The selectivity within one substrate pair is expressed as the quotient of the specificity constants (k_{cat}/K_M) for the respective arginine and lysine substrate. The change in P1 selectivity is determined by the quotient of the selectivities of the Ala190 v. Ser190 enzymes.

rigid side chain of arginine may be better suited to stabilise the S1 pocket of rf9a by an induced fit mechanism. In addition, the guanidinium group of arginine, but not the terminal amino group of lysine, is able to interact with the carbonyl of Glu219, again stabilising the S1 pocket. The latter interaction will further contribute to correctly position the substrate for a productive turnover.

The detailed analysis of the activity enhancement of rf9a-S190A towards P1-arginine substrates would require tracking the changes in the individual kinetic parameters, which was impossible in the case of rf9a. By analogy with the other studied enzymes, one would assume that the mutant rf9a-S190A has increased K_M values, and k_{cat} values dependent on the P1 residue, i.e. increased k_{cat} for P1-arginine and decreased k_{cat} for P1-lysine. Given the kinetic peculiarities of rf9a, additional mechanisms may superimpose these dependences.

3.5. The activity enhancement of rf9a-S190A reflects an evolutionary relation to rf10a

The coagulation enzymes factor IXa and factor Xa are evolutionarily closely related. Despite the large differences in the absolute reactivities of these enzymes, this relation is reflected by their parallel change in activity dependent on Ala/Ser at position 190 of the enzyme and Arg/Lys at P1. The Ala190 variant of either enzyme is twice as active as the respective Ser190 variant towards the P1-arginine substrate, while the Ala190 activity towards P1-lysine substrates is reduced by a factor of two (Table 3). Moreover, Ser190 in factor IXa is encoded by TCN, not AGY. This observation is in line with the notion that residue 190 is evolutionarily related by single site mutations to Ala190 in factor Xa, encoded by GCC [2].

The natural substrates of both factor IXa and Xa have arginine in P1 exclusively. Ala190, the more active variant, is conserved in factor Xa in all species. By contrast, factor IXa has a strictly conserved Ser190, which confers only half the activity of Ala190. Given the low absolute activity of factor IXa, the activity reduction caused by Ser190 is relatively modest. Nevertheless, Ser190 emphasises the evolutionary optimisation of factor IXa towards an enzyme which is hardly active in the absence of its cofactor and correct substrate. Ser190 may represent a choke, which is released only in the Xase complex. This underlines factor IXa's critical role in the blood coagulation cascade.

3.6. Substrate preference in dependence on Ser/Ala190 is universally conserved in serine proteases

rXYa combines properties of rf10a with those of trypsin [11]. In particular, both the S1 and S4 substrate recognition sites are derived from both factor Xa (His57, Asp102; Tyr99) and trypsin (Ser195, Ser190; 170–175 loop). Notably, the bifactorial S1–P1 dependence, reflected by the approximately five-fold selectivity enhancement for arginine versus lysine P1 residues in the presence of Ala190 versus Ser190, resembles that of evolutionarily optimised enzymes (P1 selectivity columns in Table 3). This invariance of the selectivity contrasts with the otherwise smoothened activity profile (Table 1) [11], which is caused by the hybrid nature of important substrate binding sites. The robustness of the identified cross-dependence even in an engineered hybrid enzyme emphasises its universal conservation within the serine protease family.

3.7. Biotechnological implications

The conserved cross-dependence allows us to predict the effect of a Ser/Ala substitution in position 190 on the P1-arginine over P1-lysine preference. While we now understand how to engineer an approximately five-fold improved arginine specificity in the S1 site of any serine protease (introduction of Ala190), additional mutations will be necessary to reverse the specificity towards a preference for lysine. In particular, we propose that in addition to introduction of Ser190 it would be necessary to block binding of P1-arginine and enable direct P1-lysine interaction with the protein matrix, e.g. by introduction of a glutamate at position 189.

The engineering of proteases with enhanced specificity can be valuable for example in the biotechnological production of peptides from precursor proteins, because loss due to unspecific cleavage can be minimised and the downstream purification simplified.

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